


# Hepatitis E Virus Replication and Interferon Responses in Human Placental Cells

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Hepatitis E virus (HEV) is a member of the genus *Orthohepevirus* in the family *Hepeviridae* and the causative agent of hepatitis E in humans. HEV is a major health problem in developing countries, causing mortality rates up to 25% in pregnant women. However, these cases are mainly reported for HEV genotype (gt)1, while gt3 infections are usually associated with subclinical courses of disease. The pathogenic mechanisms of adverse maternal and fetal outcome during pregnancy in HEV-infected pregnant women remain elusive. In this study, we observed that HEV is capable of completing the full viral life cycle in placental-derived cells (JEG-3). Following transfection of JEG-3 cells, HEV replication of both HEV gts could be observed. Furthermore, determination of extracellular and intracellular viral capsid levels, infectivity, and biophysical properties revealed production of HEV infectious particles with similar characteristics as in liver-derived cells. Viral entry was analyzed by infection of target cells and detection of either viral RNA or staining for viral capsid protein by immunofluorescence. HEV gt1 and gt3 were efficiently inhibited by ribavirin in placental as well as in human hepatoma cells. In contrast, interferon- $\alpha$  sensitivity was lower in the placental cells compared to liver cells for gt1 but not gt3 HEV. Simultaneous determination of interferon-stimulated gene expression levels demonstrated an efficient HEV-dependent restriction in JEG-3. **Conclusion:** We showed differential tissue-specific host responses to HEV genotypes, adding to our understanding of the mechanisms contributing to fatal outcomes of HEV infections during pregnancy. Using this cell-culture system, new therapeutic options for HEV during pregnancy can be identified and evaluated. (*Hepatology Communications* 2018;2:173-187)

## Introduction

**H**epatitis E virus (HEV) is a major cause of viral hepatitis and is classified in the genus *Orthohepevirus* and the family *Hepeviridae*.<sup>(1)</sup> Its genome contains a positive-oriented single-stranded RNA with three open reading frames (ORF), of which ORF1 encodes a multifunctional polypeptide involved in RNA replication, ORF2 the viral capsid protein, and ORF3 a nonstructural protein that can

function as a viroporin.<sup>(2,3)</sup> HEV infections in humans are caused by five genotypes (gts) that differ in their worldwide distribution, their hosts, and their route of transmission. Viruses of gt1 and gt2 are found mainly in developing countries and are transmitted by the fecal-oral route. They solely infect humans and show high epidemic potential, causing up to 120,000 symptomatic cases in a single outbreak.<sup>(4)</sup> Viruses of gt3 and gt4 cause zoonotic infections transmitted mainly by contaminated food or transfusion of contaminated

*Abbreviations:* ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FFU, focus-forming units; gt, genotype; HEV, hepatitis E virus; HEVcc, cell culture-derived infectious HEV; IFIT1/3, interferon-induced protein with tetratricopeptide repeats; IFN, interferon; ISG, interferon-stimulated gene; JAK, Janus kinase; MEM, minimum essential medium; Mx1, MX dynamin-like guanosine triphosphatase 1; NME, nucleoside diphosphate kinase; ORF, open reading frames; p.t., posttransfection; PBS, phosphate-buffered saline; PBTG, 10% goat serum, 1% bovine serum albumin, and 0.1% Triton X-100 in PBS; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RBV, ribavirin; SOF, sofosbuvir; STAT1, signal transducer and activator of transcription 1; tRNA, transfer RNA.

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blood products.<sup>(5)</sup> It has been shown that gt7 can be transmitted from infected dromedaries by undercooked flesh and milk.<sup>(6)</sup> HEV infections can cause arthralgia, vomiting, weakness, loss of appetite, abdominal pain, and hepatitis with accompanied symptoms, including jaundice and itching.<sup>(7)</sup> However, clinical symptoms occur in only 2%–5% of patients with acute HEV infection, as indicated by vaccine trials where more than 110,000 Chinese individuals were followed.<sup>(8)</sup> In apparent cases, mortality rates are estimated to be between 0.5% and 3%,<sup>(9)</sup> leading to up to 70,000 deaths worldwide per year.<sup>(10)</sup> Ribavirin (RBV) remains the treatment of choice in chronic infections and can be considered in fulminant cases.<sup>(11–13)</sup> However, RBV administration should be carefully considered because of possible side effects,<sup>(11)</sup> in particular anemia and treatment failure due to the selection of viral mutants with increased replication fitness.<sup>(14–16)</sup> Of note, RBV is also contraindicated in pregnant individuals. To date, only one prophylactic vaccine has been licensed (Hecolin),<sup>(8)</sup> which is currently not available outside of China.

One striking feature of HEV is the development of extrahepatic manifestations, including neurologic injuries, pancreatitis, acute thrombocytopenia, aplastic anemia, and renal disease.<sup>(17)</sup> Maternal mortality rates of about 25% in association with fulminant hepatic failure have been observed in HEV-infected pregnant women,<sup>(18)</sup> and the human placenta was also implicated as an extrahepatic replication site of HEV. Notably,

vertical transmission has been shown to occur frequently,<sup>(19)</sup> and adverse fetal outcome, especially in the third trimester, include preterm delivery, abortion, stillbirth, and intrauterine fetal and neonatal death.<sup>(18,20,21)</sup> However, the underlying pathologic and virologic mechanisms of an HEV gt1 infection during pregnancy remain largely undefined.

In this study, we investigated whether HEV is capable of completing the whole viral life cycle in human placental-derived cells and if there are genotype-specific replication differences. Viral replication was assessed after transfection with HEV subgenomic replicons and a full-length genomic clone. Viral assembly and release were studied by the detection of intracellular and extracellular viral capsid levels as well as by associated infectivity and subsequent viral entry by infection with gt1 and gt3 virions. In addition, we evaluated HEV susceptibility to antiviral compounds and interferon (IFN) in placental-derived cells. Collectively, the results implicate productive HEV replication in the placenta with a potent restriction of host immune responses.

## Materials and Methods

### COMPOUNDS

IFN- $\alpha$ 2a was obtained from the human IFN sampler (PBL Biomedical Laboratories, Piscataway, NJ).

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RBV was received from either Sigma-Aldrich (St. Louis, MO) or ICN Pharmaceuticals (Costa Mesa, CA). Sofosbuvir was received from [selleckchem.com](http://selleckchem.com) (Munich, Germany). All compounds were stored and diluted according to the manufacturer's recommendations.

## GAUSSIA LUCIFERASE MEASUREMENT

We measured *Gaussia* luciferase activity in supernatants of transfected cells at 4, 24, 48, and 72 hours posttransfection (p.t.) (replication kinetic assay) or 72 hours p.t. (compound dose-response assay). Briefly, 20  $\mu$ L of supernatant per well was transferred to a white, flat-bottom, 96-well microplate followed by the detection of luminescence using a microplate reader (Centro XS3 LB960; Berthold Technologies, Bad Wildbad, Germany) with coelenterazine as a substrate.

## HEV CONSTRUCTS AND *IN VITRO* TRANSCRIPTION

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, gt3; GenBank accession number JQ679013) and two constructs harboring a subgenomic HEV sequence coupled with a *Gaussia* luciferase reporter gene, of which one contains the gt1 Sar55/S17 strain (based on clone pSK-E2; GenBank accession no. AF444002, with insertion of an S17 sequence in the hypervariable region) and one the Kernow-C1 p6, were used to generate HEV *in vitro* transcripts as described.<sup>(22-24)</sup> Capping was performed using Ribom7G Cap Analog (Promega, Madison, WI).

## CELL CULTURE

The human liver cell line HepG2 and Huh7-derived S10-3 human hepatoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany). The HepG2/C3A subclone cells were cultured in Eagle's minimum essential medium (MEM) with glutamine (Invitrogen). The choriocarcinoma cell lines JEG-3 (ATCC Number HTB-36, established by serial cloning of BeWo; DNA profile similar to BeWo; produce human chorionic gonadotropin, human chorionic somatomammotropin, and progesterone; ethnicity, unknown; sex, male), BeWo (ATCC Number CCL-98, established from a malignant gestational choriocarcinoma of the fetal placenta;

gonadotropin, lactogen, and steroid secreting; ethnicity, unknown; sex, male), and JAR (ATCC Number HTB-144, directly established from a trophoblastic tumor of the placenta; genes for estrogen, progesterone, human chorionic gonadotropin, and human chorionic somatomammotropin expressed; ethnicity, Caucasian; sex, male) were cultured in Advanced MEM (Invitrogen). Supplements included 15% fetal calf serum (FCS) for BeWo cells, 10% ultra-low immunoglobulin G FCS for HepG2/C3A cells, and 10% FCS for all other cell lines, along with 2 mM L-glutamine, 1% nonessential amino acids (Invitrogen), 100  $\mu$ g/mL gentamicin (Invitrogen) for the HepG2/C3A cells, and 100  $\mu$ g/mL of streptomycin and 100 IU/mL of penicillin (Invitrogen) for all other cell lines. Additionally, 1% sodium pyruvate was added for HepG2/C3A and JEG-3 cells. HepG2 and HepG2/C3A cells were further grown on rat collagen-coated (SERVA Electrophoresis GmbH, Heidelberg, Germany) culture plates. Cells were kept at 37 °C in a 5% (volume/volume) CO<sub>2</sub> incubator.

## HEV REPLICATION ASSAY

For transfection, we used the electroporation technique in accordance with previous reports.<sup>(25)</sup> In brief,  $4-9 \times 10^6$  cells/mL in 400  $\mu$ L of Cytomix containing 2 mM adenosine triphosphate and 5 mM glutathione were mixed with 3  $\mu$ g of the respective *in vitro* transcribed HEV RNA. After electroporation with a Gene Pulser system (Bio-Rad, Munich, Germany), cells were immediately transferred into 10-15 mL of the respective culture medium. Cell suspensions were seeded into 12-well plates (1 mL per well, electroporation with subgenomic HEV RNA), 24-well plates, partly provided with glass coverslips (500  $\mu$ L per well, electroporation with full-length HEV RNA) or 96-well plates (50  $\mu$ L per well, electroporation with subgenomic HEV RNA). Four hours p.t., medium was replaced (only in 12-well and 24-well plates) and fresh culture fluid or medium containing compounds at indicated concentrations was added. At indicated time points p.t., the supernatant was collected and cells were lysed, fixed, or subjected to a freeze-thaw cycle prior to further analysis.

## DETECTION OF HEV ANTIGEN

At 4, 24, 48, and 72 hours p.t., the cell culture supernatant and cells were harvested. The cells were scraped in 1 mL of fresh culture fluid and lysed by

three freeze and thaw cycles. Subsequently, the antigen content in cell lysates and supernatants was determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (Wantai Biopharmaceuticals Inc., Beijing, China) as described.<sup>(26)</sup> In this assay, antigen (ORF2-encoded capsid protein) is captured using goat polyclonal anti-HEV antibodies, and antigen detection is visualized with enzyme-linked monoclonal antibodies against the ORF2-encoded capsid protein. We added 100  $\mu$ L of (diluted) sample to each reaction, and the assay was performed in accordance with the manufacturer's instructions.

## GENOTYPE 1 AND 3 HEV INFECTION AND DETECTION

Cell culture-derived infectious HEV (HEVcc) based on the gt3 Kernow-C1 strain was generated as described,<sup>(27)</sup> and primary isolate gt1 strain Sar55 ( $1.21 \times 10^8$  viral RNA copies/mL) in 10% fecal suspension from infected macaques was obtained from Suzanne U. Emerson (National Institutes of Health). Approximately  $5 \times 10^5$  genome equivalents of gt1 Sar55 or gt3 p6 were used to infect  $2 \times 10^4$  JEG-3 cells or S10-3 cells for 12–16 hours, followed by removal of the inoculum by thorough washes with culture medium. Seven days postinfection, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 minutes and blocked with 10% goat serum, 1% bovine serum albumin, and 0.1% Triton X-100 in PBS (PBTG buffer) at room temperature for 2 hours. Cells were incubated with anti-ORF2 in PBTG (1:200) for 2 hours at room temperature, followed by several washes with PBS. Secondary antibody conjugated to Alexa Fluor 594 (Thermo Fisher Scientific) in PBTG buffer (1:1,000) was added and incubated at room temperature for 1 hour, followed by several washes with PBS. For the determination of production of infectious particles in JEG-3 and HepG2 cells, cells were transfected as described and treated in accordance to a reported protocol.<sup>(28)</sup> Briefly, cells were electroporated as described above, incubated 6–7 days in Eagle's MEM with ultra-low immunoglobulin G FCS, and cell growth was stopped by adding hydroxyurea where necessary. Supernatant was concentrated in Amicon Ultra centrifugal filter units, molecular weight cutoff 10 kDa (Merck Millipore, Tullagreen, Ireland) to harvest extracellular virus, and cells were lysed in fresh medium by freeze and thaw cycles to harvest intracellular virus. Extracellular and intracellular virus of the same

concentration were used for infection of HepG2/C3A cells to quantify virus infectivity. These virus stocks were also used for density gradient centrifugation.

## INDIRECT IMMUNOFLUORESCENCE

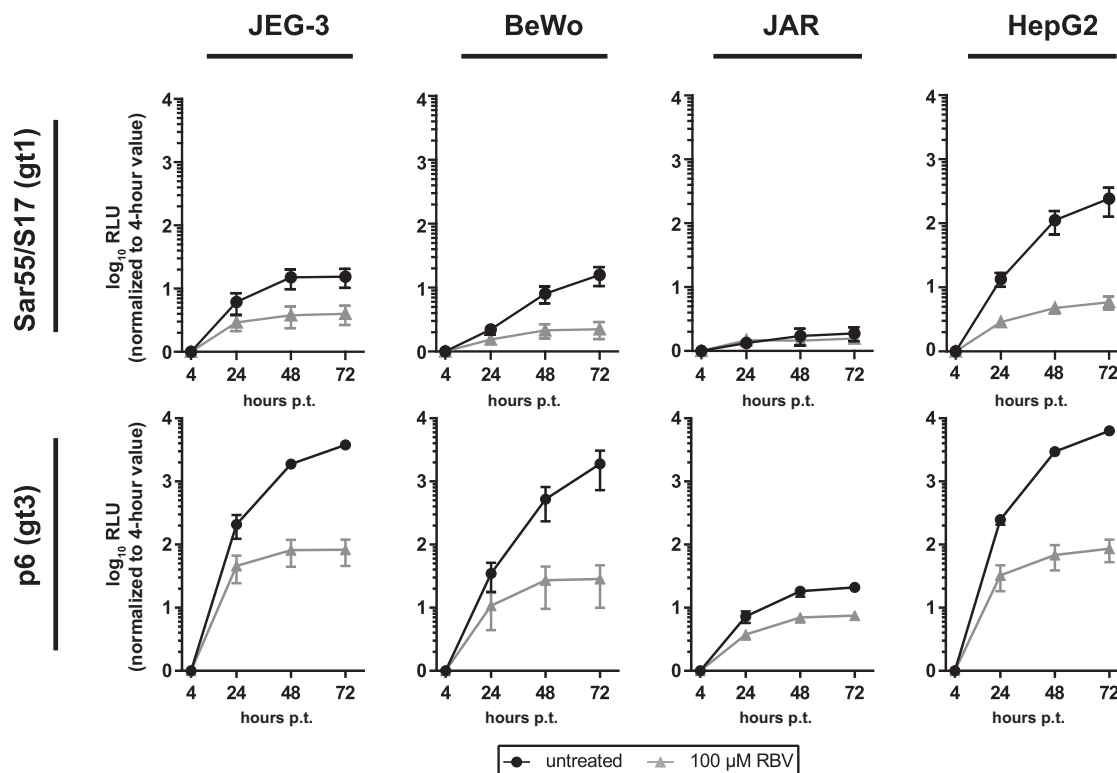
At 48 hours p.t. or 7 days postinfection, transfected cells previously seeded onto coverslips or on 96-well plates were fixed with 3% PFA in PBS and permeabilized with 0.5% Triton X-100 in PBS. After blocking of nonspecific binding with 5% goat serum (Sigma-Aldrich) and horse serum (Gibco) in PBS, immunostaining of ORF2-encoded capsid protein was performed. A 1:1,000 PBS-diluted mouse antibody directed against the ORF2-encoded protein (amino acids 434–457, clone 1E6; LifeSpan BioSciences, Inc., Seattle, WA) or a 1:500 dilution of an ORF2-specific rabbit hyperimmune serum (kindly provided by R. Ulrich, Friedrich Loeffler Institute, Greifswald, Germany) was added. A fluorescently labelled goat antibody (AlexaFluor 488; Life Technologies) was used at a dilution of 1:1,000 in PBS with 5% goat or horse serum to detect bound primary antibodies. DNA was labelled with 4',6'-diamidino-2-phenylindole (Life Technologies). For quantification of virus infectivity, wells were manually observed for specific fluorescence, and the presence of fluorescent foci was recorded. A fluorescent focus was defined as a minimum of two adjacent cells showing clear intracytoplasmic fluorescence. The number of fluorescent foci was counted in the highest dilution showing fluorescence, and the respective focus-forming units (FFU) were calculated. The limit of detection was 1 FFU.

## STATISTICS

GraphPad Prism 7.03 software was used for data analysis using either two-way analysis of variance of log-transformed values followed by Tukey's multiple comparisons test or an unpaired *t* test with Welch's correction, or repeated measures one-way analysis of variance of log-transformed values followed by Dunnett's multiple comparison test, as indicated in the figure legends. *P* < 0.05 was considered significant.

For additional information on Materials and Methods, please refer to the [Supporting Information](#) for this article.





**FIG. 1.** Replication of subgenomic HEV genotype 1 and 3 in different placental-derived cell lines. Different cell lines JEG-3, BeWo, JAR, and HepG2 were transfected by electroporation with HEV RNA of gt1 strain Sar55/S17 and gt3 strain Kernow-C1 p6 reporter constructs and were left untreated (black circles) or treated with 100  $\mu$ M RBV (gray triangles) 4 hours p.t. *Gussia* luciferase activity was assessed at 4, 24, 48, and 72 hours p.t. and normalized to 4-hour values. Depicted are the mean values of three independent experiments  $\pm$  SEM. Abbreviation: RLU, relative light unit.

## Results

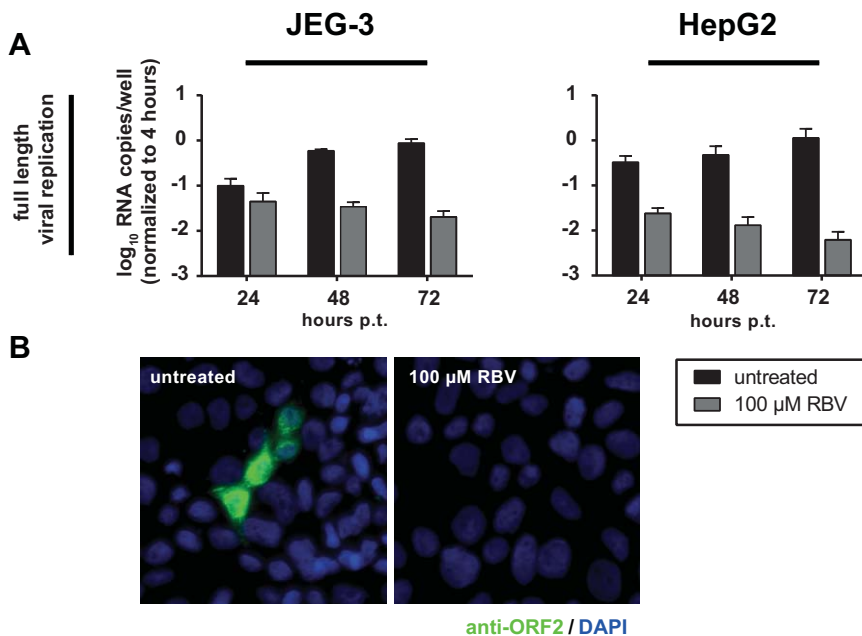
### HEV GENOTYPE 1 AND 3 STRAINS ARE REPLICATING IN PLACENTAL-DERIVED CELL LINES

Three different placental-derived choriocarcinoma cell lines, JEG-3, BeWo, and JAR, were examined for replication of HEV subgenomic reporter viruses of gt1 (strain Sar55, clone Sar55/S17) and gt3 (Kernow-C1 p6). The reporter viruses harbor a *Gussia* luciferase, which replaces parts of the ORF2 gene.<sup>(23)</sup> The human hepatocellular carcinoma cell line HepG2 was tested as a positive control in parallel, and RBV was used as an HEV RNA replication inhibitor. JEG-3 and BeWo placental-derived cells supported replication over time of HEV gt1 and of gt3 even more efficiently (Fig. 1). In both genotypes, replication was blocked by RBV.

In contrast, JAR cells displayed no productive gt1 replication and very low gt3 reporter activity (Fig. 1). The liver-derived HepG2 cells showed efficient replication of both HEV gts (Fig. 1). In summary, gt1 and gt3 HEV replicons were able to replicate in two different placental-derived cell lines.

### PLACENTAL-DERIVED JEG-3 CELLS SUPPORT FULL-LENGTH HEV REPLICATION AND PRODUCTION OF INFECTIOUS PARTICLES

Based on these results, JEG-3 cells were used in subsequent experiments for HEV replication kinetics as these cells were similar to those observed in HepG2 cells (Fig. 1). In addition, JEG-3 cells are widely accepted as a placental and trophoblastic *in vitro* model system.<sup>(29,30)</sup> We next assessed whether JEG-3 cells



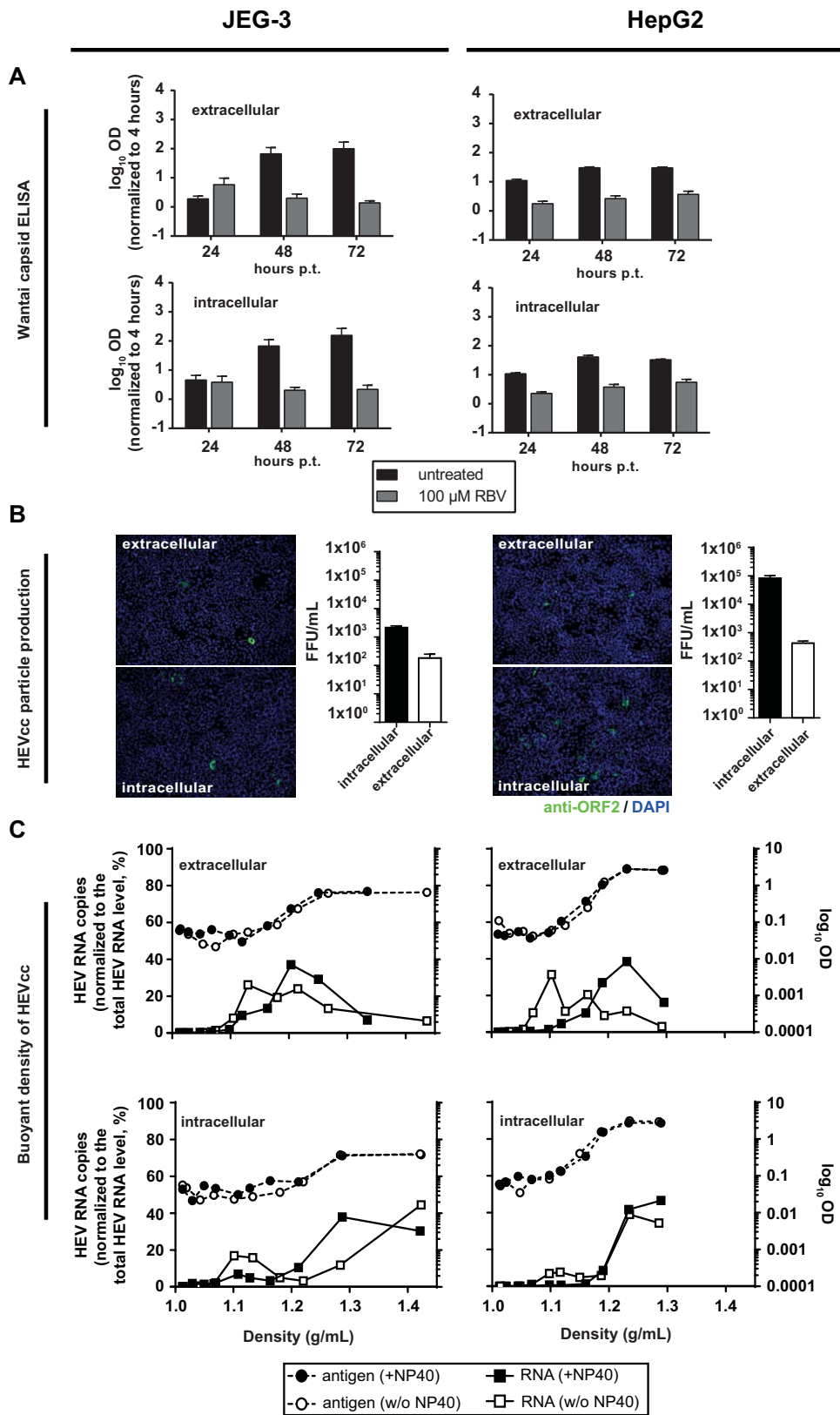
**FIG. 2.** Replication of full-length HEV and detection of intracellular and extracellular capsid protein. JEG-3 and HepG2 cells were transfected by electroporation with full-length HEV RNA gt3 Kernow-C1 p6 and were left untreated (black bars) or treated with 100  $\mu$ M RBV (gray bars) 4 hours p.t. (A) Viral replication in each cell line was measured by qRT-PCR 4, 24, 48, and 72 hours p.t. in cell lysates. (B) Immunofluorescence detection of HEV antigen in paraformaldehyde-fixed JEG-3 cells 48 hours p.t. with ORF2-encoded antigen (green) and with DAPI (blue). Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.

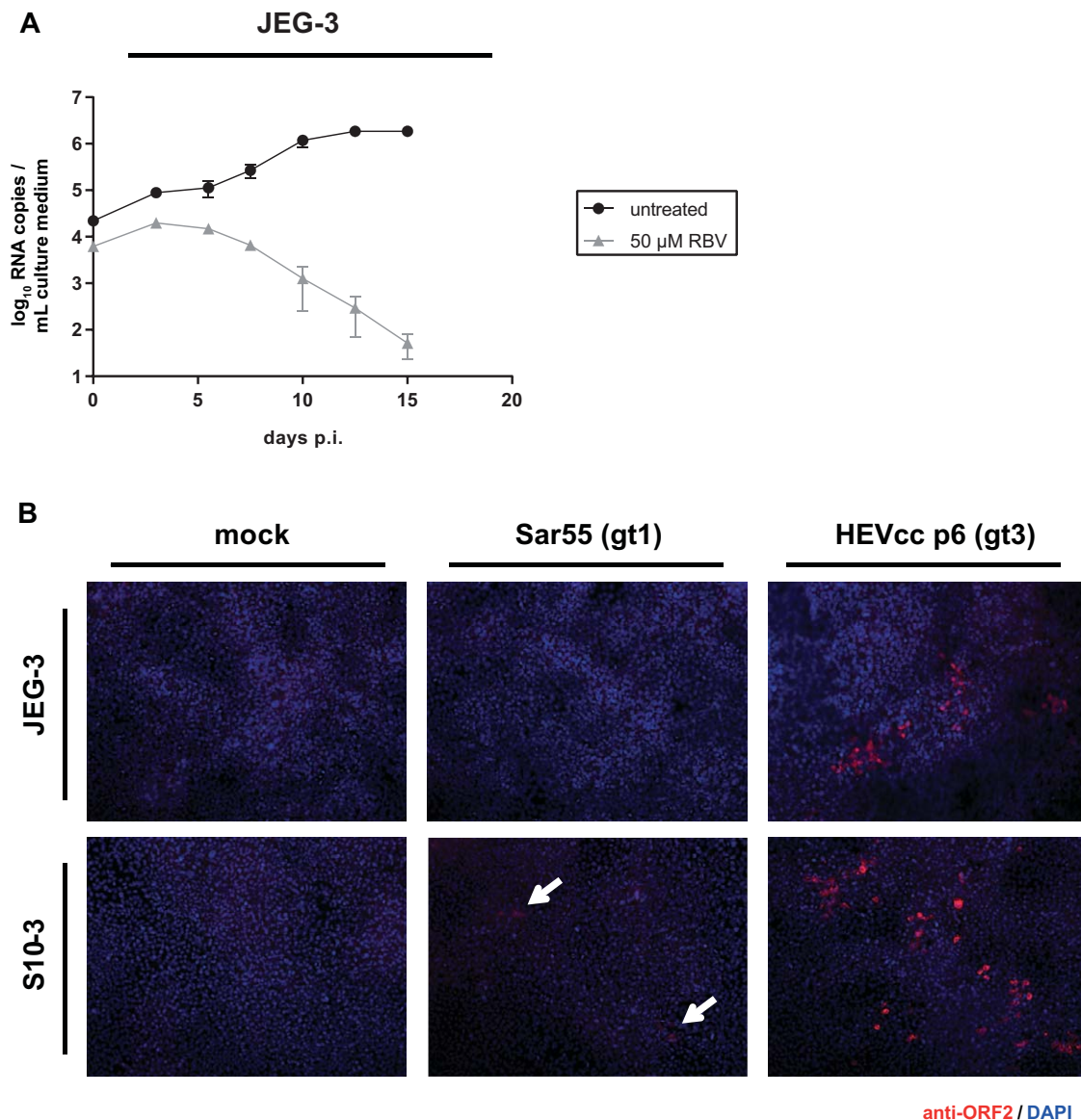
support replication of full-length HEV RNA by transfection of gt3 HEV p6 (not harboring a reporter gene) following detection by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) targeting the ORF1 gene. Accumulation of HEV RNA could be detected in JEG-3 cells at similar levels observed in HepG2 cells. In both cell lines, HEV RNA copy numbers were decreased by RBV treatment (Fig. 2A). To visualize HEV antigen expression in JEG-3 cells, immunofluorescence staining with ORF2-specific antibodies was performed. Capsid-positive cells were detectable, which was not the case for the RBV-treated cells (Fig. 2B).

Next, we examined whether placental-derived JEG-3 cells were capable of assembling and releasing HEV particles by quantification of ORF2-encoded capsid protein by specific ELISA performed on both the lysate of transfected cells (intracellular) as well as the respective supernatant (secreted) (Fig. 3A). Extracellular capsid protein increased over time in both compartments, indicating efficient virus assembly and release in both cell types (Fig. 3A). RBV treatment of transfected cells reduced both intracellular and extracellular capsid levels (Fig. 3A). The production of HEV infectious particles from JEG-3 cells in comparison to liver cells was determined as follows: The intracellular infectivity of JEG-3 cells was approximately  $2 \times 10^3$  FFU/mL, which is one order of

magnitude lower compared to the extracellular-associated infectivity (Fig. 3B, left panel). In HepG2 cells, the intracellular infectivity was higher with  $1 \times 10^5$  FFU/mL and the extracellular infectivity about 100-fold lower (Fig. 3B, right panel). To assess the difference in particle composition between placenta- and liver-derived cell lines, we performed a gradient analysis of intracellular and extracellular HEVcc, including an NP-40 detergent treatment to disrupt potential viral particle-associated membranes. For both cell types, extracellular viral RNA levels peaked at a density of approximately 1.10-1.15 g/mL (Fig. 3C). Treatment with NP-40 shifted the peak of viral RNA to a higher density, implicating a potent disruption of potential membrane structures. Intracellularly, HEV RNA levels were present at higher densities, suggesting mainly nonenveloped viral particles with minor effects of the NP-40 treatment. However, in JEG-3-derived cells, about 20% of the viral RNA was also detected at densities comparable to the extracellular particles. HEV antigen levels were also detected by the Wantai capsid ELISA for all fractions and demonstrated antigen levels at density fractions that were not associated with HEV RNA (Fig. 3C). Collectively, JEG-3 placental-derived cells supported full-length RNA replication, viral assembly, and release and the production of infectious particles with similar biophysical properties.

**FIG. 3.** Determination of HEV infectious particle production, infectivity, and biophysical properties of HEVcc. JEG-3 and HepG2 cells were transfected by electroporation with full-length authentic HEV RNA gt3 Kernow-C1 p6. (A) Cells were left untreated (black bars) or treated with 100  $\mu$ M RBV (gray bars) 4 hours p.t. A commercially available HEV antigen ELISA detecting the ORF2-encoded capsid protein was used to determine extracellular and intracellular capsid protein amounts over time. All values were normalized to 4-hour values. Depicted are the mean values of three independent experiments  $\pm$  SEM. (B) Intracellular (black bars) and extracellular (white bars) virus produced in JEG-3 and HepG2 cells was harvested and used to infect HepG2/C3A cells. Infected cells were stained for ORF2-specific antigen (green) and DAPI (blue) 7 days postinfection. Quantitative infectivity was determined manually by counting infectious foci. (C) Iodixanol density gradients of extracellular and intracellular HEVcc produced in JEG-3 and HepG2 cells either treated with the detergent NP-40 for 1 hour (black) or left untreated (white). Fractions were analyzed for buoyant density with a refractometer for HEV antigen (circles) and HEV RNA (boxes). All antigen values below the cut-off value  $\log_{10}$  OD 0.202 are reported as negative by the ELISA. RNA levels are depicted as percentage of total RNA level. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; OD, optical density.





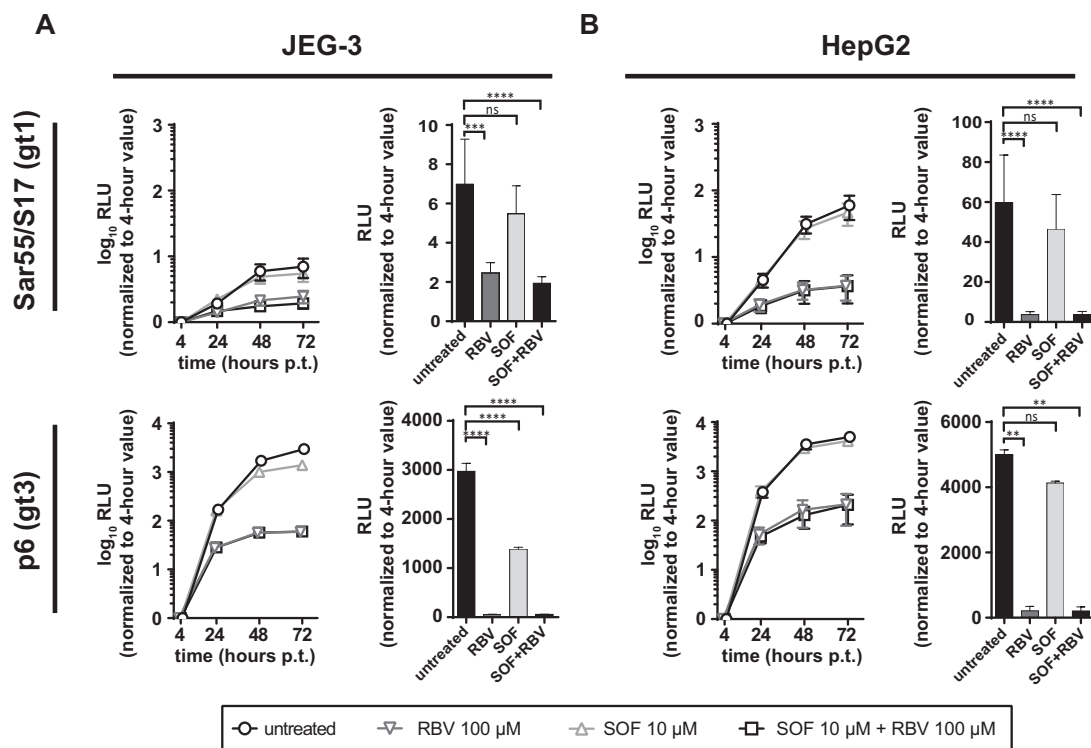
**FIG. 4.** Infection of JEG-3 cells with HEV gt3 and gt1. (A) JEG-3 cells were infected with HEVcc originating from the gt3 p6 strain and were left untreated (black circles) or incubated with 50  $\mu$ M RBV (gray triangles). Samples were taken every 2–3 days, and viral RNA was measured by qRT-PCR. Depicted are the mean values of three independent experiments  $\pm$  SEM. (B) Immunofluorescence staining of JEG-3 or S10-3 cells infected with gt1 Sar55 primary stool suspension or HEVcc gt3 p6-derived particles. Cells were stained for HEV capsid ORF2 (red) or with DAPI (blue) 7 days postinfection. White arrows indicate HEV-positive cells. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole; p.i., postinfection.

## JEG-3 CELLS ARE PERMISSIVE FOR INFECTION WITH HEVcc

To evaluate if JEG-3 cells allowed HEV entry and replication, cells were infected with HEVcc and viral RNA released over time was detected in the cell culture supernatants as described for human liver cells<sup>(12,25)</sup> in comparison to RBV-treated cells. An

increase in viral RNA postinfection was observed, and this reached 6.27 log<sub>10</sub> RNA copies/mL in the culture medium, indicating the productive infection of JEG-3 (Fig. 4A). Treatment with 50  $\mu$ M RBV was able to efficiently inhibit HEV replication after infection (decrease of 4.5 log<sub>10</sub> RNA copies). Because infection with cell culture-grown HEV gt1 particles is not yet possible, we next performed infection of JEG-3 cells



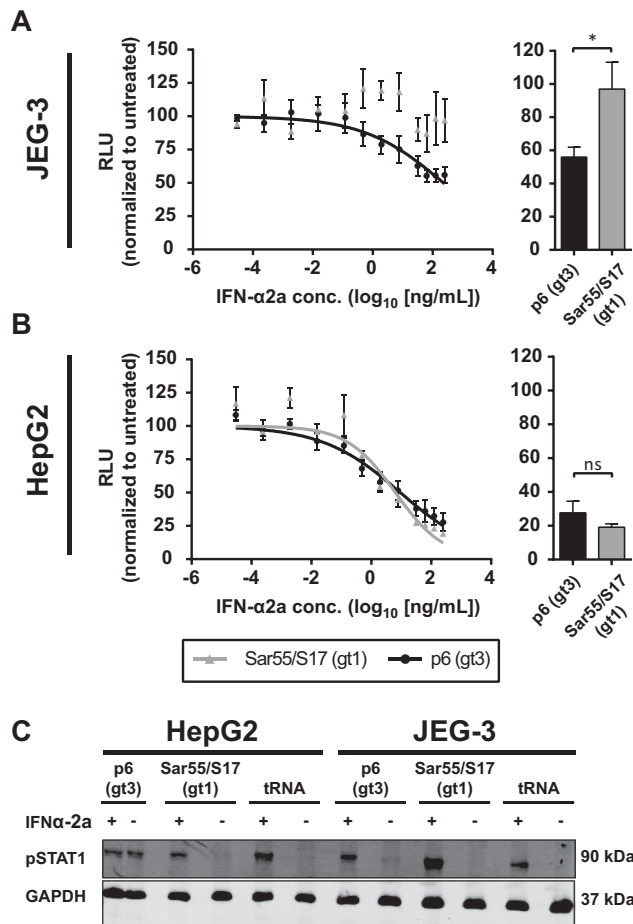


**FIG. 5.** Antiviral activity of sofosbuvir in placental-derived JEG-3 cells. (A) JEG-3 and (B) HepG2 cells were transfected by electroporation with HEV RNA of gt1 strain Sar55/S17 and gt3 strain Kernow-C1 p6 reporter constructs. Cells were treated with either 100 μM RBV (gray inverted triangles), 10 μM SOF (gray triangles), or with a combination of 10 μM SOF and 100 μM RBV (dark gray boxes) 4 hours p.t. *Gaussia* luciferase activity was determined at 4, 24, 48, and 72 hours p.t. and normalized to 4-hour values. Bar plots of data points representing the luciferase activity 72 hours p.t. for each cell line and gt illustrate the maximum replication inhibition. Depicted are the mean values of at least three independent experiments  $\pm$  SEM (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ; repeated measures one-way analysis of variance of log-transformed values followed by Dunnett's multiple comparison test). Abbreviations: ns, not significant; RLU, relative light unit.

with a gt1 Sar55 primary isolate stool suspension not harboring the S17 insertion derived from infected rhesus macaques.<sup>(31)</sup> Further controls included HEVcc gt3 particles and infection of Huh-7 human hepatoma-derived cell line S10-3, which is highly permissive for HEVcc replication.<sup>(31)</sup> Challenged cell lines were stained for ORF2-encoded antigen 7 days postinfection. The gt1 primary isolate infection resulted in only a few ORF2-positive S10-3 cells; these cells were not seen with the JEG-3 cells (Fig. 4B). Anti-ORF2-positive cells could readily be detected in JEG-3 and S10-3 cells after gt3 HEVcc infection, confirming the previous results (Fig. 4B). Together, gt1-derived primary HEV infection efficiency was limited in human liver cells and not detectable in placental-derived cells. However, JEG-3 cells supported viral entry and productive replication of gt3 HEVcc detected by qRT-PCR and immunofluorescence.

## ANTIVIRAL PROPERTIES OF SOFOSBUVIR AND IFN- $\alpha$ 2a IN JEG-3 CELLS

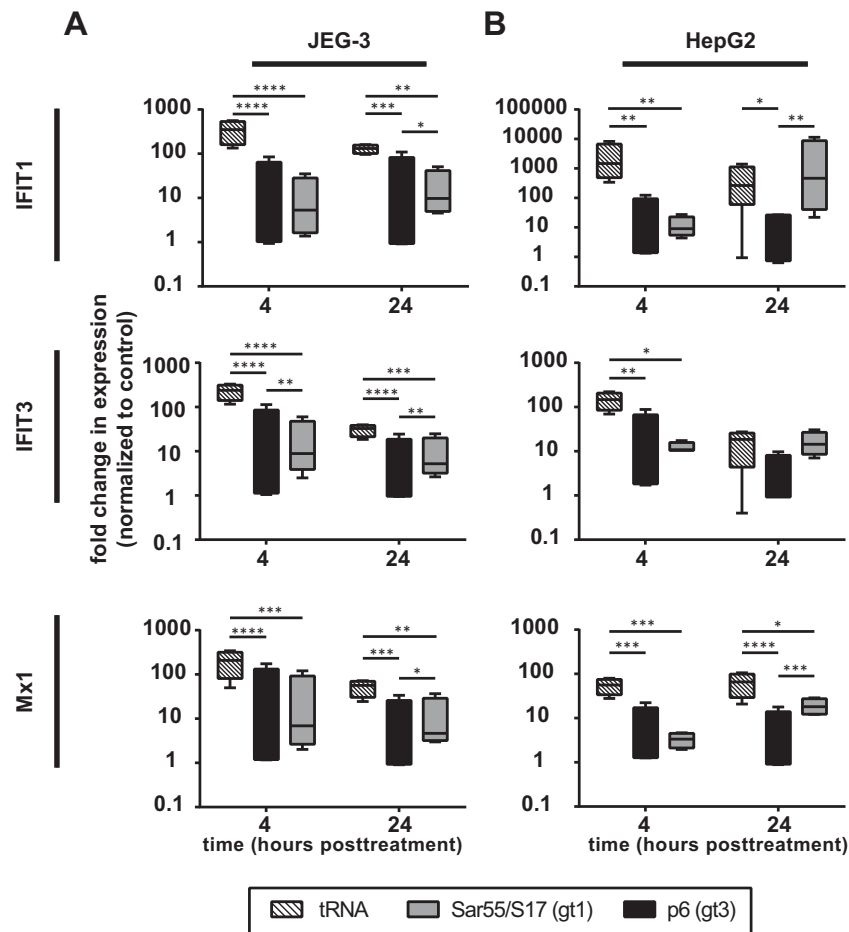
Although no approved drugs for HEV infections are available, IFN- $\alpha$ 2a and RBV have been used as off-label medications in chronically infected patients. Recently, sofosbuvir (SOF), a direct-acting antiviral inhibiting the NS5B protein of hepatitis C virus,<sup>(32)</sup> has been shown to exert antiviral activity against HEV.<sup>(27)</sup> Therefore, we evaluated the effect of these drugs in extrahepatic placental cells replicating HEV gt1 and gt3. SOF treatment alone showed no significant inhibition of gt1 replication in placental-derived (Fig. 5A) and liver-derived cells (Fig. 5B). Gt3 replicons were significantly inhibited 72 hours p.t. by SOF in JEG-3 cells but only moderately affected in HepG2 cells, and RBV as well as the combination of both



**FIG. 6.** Antiviral properties of IFN- $\alpha$ 2a and analysis of STAT1 phosphorylation after treatment. (A) JEG-3 or (B) HepG2 cells were transfected by electroporation with HEV RNA of gt1 strain Sar55/S17 (light gray triangles and bars) and gt3 strain Kernow-C1 p6 (dark gray circles and bars) reporter constructs and treated with serially diluted IFN- $\alpha$ 2a 4 hours p.t. *Gaussia* luciferase counts were assessed 72 hours p.t. and normalized to untreated control values. Side by side plotting of the data points representing the highest IFN concentration (250 ng/mL) illustrates the maximum replication inhibition (A and B, right panels). Depicted are the mean values of four independent experiments  $\pm$  SEM (\* $P$  < 0.05; unpaired  $t$  test with Welch's correction). (C) Western blot-based analysis of STAT1 phosphorylation in JEG-3 and HepG2 cells after transfection with HEV RNA of gt1 strain Sar55/S17, gt3 strain Kernow-C1 p6 reporter constructs, and tRNA. Cells were either treated with 1,000 IU IFN- $\alpha$ 2a 12 hours p.t. for 30 minutes or left untreated and were lysed directly afterward. Depicted are the 90-kDa bands for pSTAT1 as well as 37-kDa bands for GAPDH (one out of two independent experiments shown). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ns, not significant; pSTAT1, phosphorylated STAT1; RLU, relative light unit.

moderate dose-dependent inhibition of gt3 replication up to 50%; however, no effect of IFN on gt1-dependent replication of the Sar55/S17 replicon was observed (Fig. 6A). A comparison of reporter activity of both gts at the highest IFN dose of 250 ng/mL illustrates the higher susceptibility of the gt3 replicon to IFN- $\alpha$ 2a (Fig. 6A, right panel). In contrast, in HepG2 cells a dose-dependent reduction in HEV replication efficiency was noted for both strains (Fig. 6B), indicating a lower IFN sensitivity of the gt1 strain in the placental-derived cells. To exclude defects in the cellular innate immune response of the placental-derived cells or impairment in activating the SOF pro-drug, we compared expression profiles of genes involved in IFN signaling and sensing between JEG-3 cells and primary human trophoblasts by differential expression analysis of a published RNA sequencing data set<sup>(30)</sup> (Supporting Fig. S1). SOF is metabolized to its active triphosphate form through hydrolysis of the carboxylate ester by either cathepsin A or carboxylesterase 1, followed by cleavage of the phosphoramidate by histidine triad nucleotide-binding protein 1, and subsequently repeatedly phosphorylated by nucleoside diphosphate kinase 1 and 2 (NME1/2).<sup>(33)</sup> Three transcripts of genes involved in IFN signaling (IFN gamma receptor 1 [IFNGR1], interleukin 10 receptor subunit beta [IL10RB], and signal transducer and activator of transcription 1 [STAT1]) and two transcripts of genes involved in SOF activation (NME1, NME1-NME2) are significantly differentially expressed (Supporting Fig. S1A). Their raw expression values in reads per kilobase of exon model per million mapped reads, however, are in ranges considered as tissue specifically expressed (Supporting Fig. S1B). Furthermore, the majority of genes involved in type I IFN signaling and sensing (IFN alpha and beta receptor subunit [IFNAR1, IFNAR2], IFNGR2, IFN lambda receptor 1 [IFNLR1], IFN regulatory factor 9 [IRF9], Janus kinase [JAK1, JAK2], STAT2, and tyrosine kinase 2) showed a similar expression level in both primary human trophoblasts as well as JEG-3 cells, suggesting a comparable cellular response to exogenous IFN- $\alpha$ 2a treatment. Next, we performed western blot analysis of the phosphorylation of STAT1 at its Y701 phosphorylation site in both cell lines replicating HEV gt3 and gt1 as well as transfer RNA (tRNA) as control. Our objective was to investigate whether the lower sensitivity of the gt1 strain in JEG-3 cells is due to a permanent phosphorylation of STAT1 in this cell line. HepG2 cells transfected with the gt3 replicon show phosphorylation of STAT1 without exogenous IFN

demonstrated high antiviral activities in both cell lines (Fig. 5A,B). We next determined the susceptibility of HEV gts to IFN- $\alpha$ 2a in JEG-3 cells. There was a



**FIG. 7.** HEV restriction in expression of selected ISGs under treatment with IFN- $\alpha$ 2a. (A) JEG-3 and (B) HepG2 cells were transfected by electroporation with HEV RNA of gt1 strain Sar55/S17 (light gray bars), gt3 strain Kernow-C1 p6 (dark gray bars) reporter constructs, or tRNA as control (hatched bars), following treatment with 1,000 IU/mL (ca. 2.6 ng/mL) IFN- $\alpha$ 2a for 24 hours. Respective cell lines were lysed and expression of depicted ISGs was assessed by qRT-PCR 4 hours and 24 hours posttreatment. Values were normalized to untreated control values. Depicted are the mean values of four independent experiments  $\pm$  SEM (\* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001; two-way analysis of variance of log-transformed values followed by Tukey's multiple comparisons test).

treatment, whereas after transfection with the gt1 replicon or control tRNA, phosphorylation occurs only after exogenous IFN treatment (Fig. 6C). This effect might be due to endogenous activation of upstream RNA-sensing mechanisms because replication efficiency is highest in HepG2 cells transfected with the gt3 replicon. In JEG-3 cells, phosphorylation of STAT1 can be observed only after exogenous IFN administration for all three conditions (Fig. 6C), suggesting activation of JAK-STAT signaling following treatment with IFN in these cells. In summary, differences in the IFN sensitivity specifically for HEV gt1 were noted between JEG-3 and HepG2 cells, and these might

play a role in the pathogenic mechanisms observed in HEV infections during pregnancy.

## INTERFERON-STIMULATED GENE EXPRESSION IS DOWN-REGULATED BY HEV IN PLACENTAL-DERIVED JEG-3 CELLS

As different studies have reported that HEV is able to counteract the IFN-stimulated gene (ISG) response in human liver cells,<sup>(34,35)</sup> we next investigated the viral restriction of the IFN pathway by determination of

ISG expression after HEV transfection in placental-derived cells. JEG-3 (Fig. 7A) and HepG2 (Fig. 7B) cells were transfected with gt1 and gt3-based subgenomic HEV constructs or a tRNA transfection control. We treated 24-hour p.t. cells with IFN- $\alpha$ 2a. Four or 24 hours posttreatment, cells were lysed and messenger RNA levels of the ISGs IFN-induced protein with tetratricopeptide repeats (IFIT1, IFIT3) and MX dynamin-like guanosine triphosphatase 1 (Mx1) were assessed with qRT-PCR and normalized to untreated cells. Both HEV strains blocked IFN type I responses by a significant down-regulation of the three measured ISGs 4 hours and 24 hours posttreatment in JEG-3 cells (Fig. 7A). Notably, the gt1 strain, which replicated less efficiently in JEG-3 cells compared to the gt3 strain (Fig. 1), displayed a similar potency in viral restriction of IFIT1 and Mx1 4 hours posttreatment. In HepG2 cells, a comparable down-regulation could be observed, particularly for IFIT3 4 hours posttreatment and Mx1, which were induced about 50-fold to 100-fold in the control cells 4 hours posttreatment (Fig. 7B). In conclusion, HEV gts were able to exert a potent anti-IFN response in the placental-derived cells.

## Discussion

The course of HEV infection during pregnancy has a more severe outcome compared to other populations and can lead to fulminant hepatitis, increasing maternal and fetal mortality.<sup>(36)</sup> However, the pathogenic mechanisms responsible for these fatal outcomes remain elusive. In this study, we characterized human placental-derived cells for their permissiveness to HEV replication and analyzed antiviral mechanisms by drug treatment as well as host innate immune responses. By the detection of HEV negative-strand RNA and antigen using immunohistochemistry in placental tissue sections of infected pregnant woman, extrahepatic replication in the placenta has been postulated.<sup>(37)</sup> We report here that the placental-derived cell line JEG-3 supported replication of gt1 and gt3 with efficiency similar to human liver cells (Figs. 1 and 2). The reason for the lower replication rate of gt1 and gt3 in placenta-derived JAR cells compared to JEG-3 and BeWo cells is currently unclear; however, this could be due to a differential host factor expression required for efficient HEV replication. The first HEV outbreak with reported cases of symptomatic pregnant women was documented in 1987 in Nepal,<sup>(38)</sup> and most of the

recent gt1 outbreaks were detected in refugee camps in developing countries, including Somalia,<sup>(39)</sup> Kenya,<sup>(40)</sup> Sudan,<sup>(41)</sup> and Uganda.<sup>(9)</sup> There are, in addition, reports mainly from Egypt in which gt1 infections during pregnancy were not associated with morbidity.<sup>(42-44)</sup> Importantly, a few cases of gt3 infection during pregnancy were also observed in industrialized countries, for example, in a study from the United Kingdom,<sup>(45)</sup> France,<sup>(46)</sup> and Germany.<sup>(47)</sup> Nevertheless, these cases do not indicate a comparable severe outcome of gt3 infections during pregnancy. Of note, high viral loads of HEV during pregnancy have been implicated as one of the factors responsible for the severity of the infection during pregnancy.<sup>(48,49)</sup> JEG-3 cells allowed HEV viral particle production with membrane-associated extracellular viral particles and biophysical properties comparable to human liver cell-derived viruses (Fig. 3). HEV antigen was determined by a commercially available ELISA and revealed capsid protein also in fractions where no viral RNA was detected. These results are in line with a recent study by Montpellier et al.<sup>(50)</sup> that identified three different forms of the viral capsid whereby two of these ORF2 forms are not associated with infectious particles. In addition, we could previously show that HEV antigen in sera from HEV-infected patients was detectable throughout all fractions of the gradients, suggesting the presence of distinct fragments of the viral capsid proteins with different densities.<sup>(26)</sup> Importantly, JEG-3 cells allowed entry and spread of HEV gt3 strain p6 (Fig. 4). The infection with primary material of the Sar55 genotype 1 isolate was restricted to human liver cells, but the infection efficiency was limited. Recently, Nair et al.<sup>(51)</sup> described a novel protein, termed ORF4, which is gt1 specific and implicated in the endoplasmic stress response promoting viral replication. If ORF4 is expressed during pregnancy in HEV infection *in vivo* and how this protein interacts with the host response require future investigation. Using the JEG-3 replication system described herein may facilitate future advances in understanding this important phenomenon. Similar to our reference hepatoma cell line HepG2, RBV efficiently inhibited HEV replication in placental-derived JEG-3 cells. As placental cells are also likely a site of extrahepatic viral replication and RBV is contraindicated in case of pregnancy, this cell model could be used as a tool for the identification and evaluation of new antivirals for HEV infection in this cohort. SOF has previously been shown to lower HEV replication and in combination with RBV demonstrated an additive effect.<sup>(27)</sup> Here, we observed



moderate effects of SOF in both cell lines, and gt1 replication seemed less sensitive to inhibition by SOF, probably due to the low replication efficiency of the gt1-based Sar55/S17 replicon.

Alteration of the immune system during hormonal changes in pregnancy has been discussed as another potential pathogenic mechanism in HEV infection,<sup>(36,42)</sup> but the described alterations mostly affect cellular immunity. According to McConkey and colleagues,<sup>(30)</sup> two-dimensional-cultured JEG-3 cells secrete only small amounts of beta-human chorionic gonadotropin, and production of human placental lactogen was also reduced, limiting studies of endogenous hormone-driven influences of HEV infection on the IFN response. However, the already moderate inhibition of HEV replication by IFN- $\alpha$  in human liver cell lines<sup>(34)</sup> was even weaker in the placental-derived cell line selective for gt1 replication (Fig. 6), while both HEV gts were able to efficiently down-regulate selected ISGs in the liver- and placental-derived cell line (Fig. 7). These differences in placenta-specific innate immune responses might be a relevant factor for the clinical features of HEV during pregnancy, and future studies are required to dissect the underlying mechanism of this phenomenon. One possible hypothesis could be differential interaction with host factors influencing IFN responsiveness. As HEV genotyping is not always routinely performed, *in vivo* evidence to support this hypothesis has not been acquired. Animal model systems to recapitulate HEV infection during pregnancy are further limited. Inoculation of pregnant rhesus monkeys with HEV gt1 could not detect differences when comparing with nonpregnant monkeys and failed to reproduce the elevated mortality rates seen in pregnant women or the development of the reported severe and fulminant hepatitis E.<sup>(52)</sup> Recently, experimental infection of pregnant rabbits with HEV demonstrated high mortality and vertical transmission. However, pathogenic mechanisms have not been addressed and are difficult to compare with the course of infection in HEV-infected pregnant women.<sup>(53)</sup>

In conclusion, we found that HEV was capable of performing the full viral life cycle in a placental-derived cell line expanding HEV tissue tropism. Further, these data revealed differential tissue-specific host responses to HEV genotypes, implicating a mechanistic role in the severe outcome of HEV infection during pregnancy.

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## REFERENCES

- 1) Smith DB, Simmonds P, Jameel S, Emerson SU, Harrison TJ, Meng XJ, et al.; International Committee on Taxonomy of Viruses Hepeviridae Study Group. Consensus proposals for classification of the family Hepeviridae. *J Gen Virol* 2014;95(Pt.10): 2223-2232.
- 2) Cao D, Meng XJ. Molecular biology and replication of hepatitis E virus. *Emerg Microbes Infect* 2012;1:e17.
- 3) Ding Q, Heller B, Capuccino JM, Song B, Nimgaonkar I, Hrebikova G, et al. Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles. *Proc Natl Acad Sci U S A* 2017;114:1147-1152.
- 4) Khuroo MS, Khuroo MS, Khuroo NS. Hepatitis E: discovery, global impact, control and cure. *World J Gastroenterol* 2016;22: 7030-7045.
- 5) Behrendt P, Steinmann E, Manns MP, Wedemeyer H. The impact of hepatitis E in the liver transplant setting. *J Hepatol* 2014;61:1418-1429.
- 6) Lee GH, Tan BH, Teo EC, Lim SG, Dan YY, Wee A, et al. Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk. *Gastroenterology* 2016;150:355-357.e353.
- 7) Hamid SS, Atiq M, Shehzad F, Yasmeen A, Nissa T, Salam A, et al. Hepatitis E virus superinfection in patients with chronic liver disease. *Hepatology* 2002;36:474-478.
- 8) Zhu F-C, Zhang J, Zhang X-F, Zhou C, Wang Z-Z, Huang S-J, et al. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. *Lancet* 2010;376:895-902.
- 9) Teshale EH, Hu DJ, Holmberg SD. The two faces of hepatitis E virus. *Clin Infect Dis* 2010;51:328-334.
- 10) Rein DB, Stevens GA, Theaker J, Wittenborn JS, Wiersma ST. The global burden of hepatitis E virus genotypes 1 and 2 in 2005. *Hepatology* 2012;55:988-997.
- 11) Dalton HR, Kamar N. Treatment of hepatitis E virus. *Curr Opin Infect Dis* 2016;29:639-644.
- 12) Debing Y, Emerson SU, Wang Y, Pan Q, Balzarini J, Dallmeier K, et al. Ribavirin inhibits *in vitro* hepatitis E Virus replication through depletion of cellular GTP pools and is moderately synergistic with alpha interferon. *Antimicrob Agents Chemother* 2014;58:267-273.
- 13) Pischke S, Hardtke S, Bode U, Birkner S, Chatzikyrkou C, Kauffmann W, et al. Ribavirin treatment of acute and chronic hepatitis E: a single-centre experience. *Liver Int* 2013;33:722-726.
- 14) Debing Y, Gisa A, Dallmeier K, Pischke S, Bremer B, Manns M, et al. A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. *Gastroenterology* 2014;147: 1008-1011.e1007.

- 15) **Todt D, Gisa A**, Radonic A, Nitsche A, Behrendt P, Suneetha PV, et al. In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome. *Gut* 2016;65:1733-1743.
- 16) Todt D, Walter S, Brown RJ, Steinmann E. Mutagenic effects of ribavirin on hepatitis E virus—viral extinction versus selection of fitness-enhancing mutations. *Viruses* 2016;8 pii:E283.
- 17) Pischke S, Hartl J, Pas SD, Lohse AW, Jacobs BC, Van der Eijk AA. Hepatitis E virus: infection beyond the liver? *J Hepatol* 2017;66:1082-1095.
- 18) Kumar A, Beniwal M, Kar P, Sharma JB, Murthy NS. Hepatitis E in pregnancy. *Int J Gynaecol Obstet* 2004;85:240-244.
- 19) Khuroo MS, Kamili S, Jameel S. Vertical transmission of hepatitis E virus. *Lancet* 1995;345:1025-1026.
- 20) Khuroo MS, Teli MR, Skidmore S, Sofi MA, Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. *Am J Med* 1981;70:252-255.
- 21) Patra S, Kumar A, Trivedi SS, Puri M, Sarin SK. Maternal and fetal outcomes in pregnant women with acute hepatitis E virus infection. *Ann Intern Med* 2007;147:28-33.
- 22) Nguyen HT, Shukla P, Torian U, Faulk K, Emerson SU. Hepatitis E virus genotype 1 infection of swine kidney cells in vitro is inhibited at multiple levels. *J Virol* 2014;88:868-877.
- 23) Shukla P, Nguyen HT, Faulk K, Mather K, Torian U, Engle RE, et al. Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* 2012;86:5697-5707.
- 24) **Shukla P, Nguyen HT**, Torian U, Engle RE, Faulk K, Dalton HR, et al. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* 2011;108:2438-2443.
- 25) Drave SA, Debing Y, Walter S, Todt D, Engelmann M, Friesland M, et al. Extra-hepatic replication and infection of hepatitis E virus in neuronal-derived cells. *J Viral Hepat* 2016; 23:512-521.
- 26) Behrendt P, Bremer B, Todt D, Brown RJ, Heim A, Manns MP, et al. Hepatitis E virus (HEV) ORF2 antigen levels differentiate between acute and chronic HEV infection. *J Infect Dis* 2016;214:361-368.
- 27) Dao Thi VL, Debing Y, Wu X, Rice CM, Neyts J, Moradpour D, et al. Sofosbuvir inhibits hepatitis E virus replication in vitro and results in an additive effect when combined with ribavirin. *Gastroenterology* 2016;150:82-85.e84.
- 28) Farcet MR, Lackner C, Antoine G, Rabel PO, Wieser A, Flicker A, et al. Hepatitis E virus and the safety of plasma products: investigations into the reduction capacity of manufacturing processes. *Transfusion* 2016;56:383-391.
- 29) **Orendi K, Kivity V**, Sammar M, Grimpel Y, Gonen R, Meiri H, et al. Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. *Placenta* 2011; 32(Suppl.):S49-S54.
- 30) McConkey CA, Delorme-Axford E, Nickerson CA, Kim KS, Sadvovsky Y, Boyle JP, et al. A three-dimensional culture system recapitulates placental syncytiotrophoblast development and microbial resistance. *Sci Adv* 2016;2:e1501462.
- 31) Emerson SU, Nguyen H, Graff J, Stephany DA, Brockington A, Purcell RH. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol* 2004;78:4838-4846.
- 32) Sofia MJ, Bao D, Chang W, Du J, Nagarathnam D, Rachakonda S, et al. Discovery of a beta-d-2'-deoxy-2'-alpha-fluoro-2'-beta-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. *J Med Chem* 2010;53: 7202-7218.
- 33) Bhatia HK, Singh H, Grewal N, Natt NK. Sofosbuvir: a novel treatment option for chronic hepatitis C infection. *J Pharmacol Pharmacother* 2014;5:278-284.
- 34) Todt D, Francois C, Anggakusuma, Behrendt P, Engelmann M, Kneegendorf L, et al. Antiviral activities of different interferon types and subtypes against hepatitis E virus replication. *Antimicrob Agents Chemother* 2016;60:2132-2139.
- 35) Nan Y, Yu Y, Ma Z, Khattar SK, Fredericksen B, Zhang YJ. Hepatitis E virus inhibits type I interferon induction by ORF1 products. *J Virol* 2014;88:11924-11932.
- 36) Perez-Gracia MT, Suay-Garcia B, Mateos-Lindemann ML. Hepatitis E and pregnancy: current state. *Rev Med Virol* 2017; doi: 10.1002/rmv.1929.
- 37) Bose PD, Das BC, Hazam RK, Kumar A, Medhi S, Kar P. Evidence of extrahepatic replication of hepatitis E virus in human placenta. *J Gen Virol* 2014;95(Pt.6):1266-1271.
- 38) Asher LV, Innis BL, Shrestha MP, Ticehurst J, Baze WB. Virus-like particles in the liver of a patient with fulminant hepatitis and antibody to hepatitis E virus. *J Med Virol* 1990;31:229-233.
- 39) Bile K, Isse A, Mohamud O, Allebeck P, Nilsson L, Norder H, et al. Contrasting roles of rivers and wells as sources of drinking water on attack and fatality rates in a hepatitis E epidemic in Somalia. *Am J Trop Med Hyg* 1994;51:466-474.
- 40) Mast EE, Polish LB, Favorov MO, Khudyakova NS, Collins C, Tukei PM, et al.; the Somali Refugee Medical Team. Hepatitis E among refugees in Kenya: minimal apparent person-to-person transmission, evidence for age-dependent disease expression, and new serologic assays. In: Nishioka K, Suzuki H, Mishiro S, Oda T, eds. *Viral Hepatitis and Liver Disease*. Tokyo, Japan: Springer; 1994:375-378.
- 41) Boccia D, Guthmann JP, Klovstad H, Hamid N, Tatay M, Ciglenecki I, et al. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. *Clin Infect Dis* 2006;42:1679-1684.
- 42) Navaneethan U, Al Mohajer M, Shata MT. Hepatitis E and pregnancy: understanding the pathogenesis. *Liver Int* 2008;28: 1190-1199.
- 43) Stoszek SK, Abdel-Hamid M, Saleh DA, El Kafrawy S, Narooz S, Hawash Y, et al. High prevalence of hepatitis E antibodies in pregnant Egyptian women. *Trans R Soc Trop Med Hyg* 2006; 100:95-101.
- 44) Stoszek SK, Engle RE, Abdel-Hamid M, Mikhail N, Abdel-Aziz F, Medhat A, et al. Hepatitis E antibody seroconversion without disease in highly endemic rural Egyptian communities. *Trans R Soc Trop Med Hyg* 2006;100:89-94.
- 45) Andersson MI, Hughes J, Gordon FH, Ijaz S, Donati M. Of pigs and pregnancy. *Lancet* 2008;372:1192.
- 46) **Anty R, Ollier L**, Peron JM, Nicand E, Cannavo I, Bongain A, et al. First case report of an acute genotype 3 hepatitis E infected pregnant woman living in South-Eastern France. *J Clin Virol* 2012;54:76-78.
- 47) **Tabatabai J, Wenzel JJ**, Soboletzki M, Flux C, Navid MH, Schnitzler P. First case report of an acute hepatitis E subgenotype 3c infection during pregnancy in Germany. *J Clin Virol* 2014;61:170-172.
- 48) Bose PD, Das BC, Kumar A, Gondal R, Kumar D, Kar P. High viral load and deregulation of the progesterone receptor signaling pathway: association with hepatitis E-related poor pregnancy outcome. *J Hepatol* 2011;54:1107-1113.
- 49) Borkakoti J, Hazam RK, Mohammad A, Kumar A, Kar P. Does high viral load of hepatitis E virus influence the severity and prognosis of acute liver failure during pregnancy? *J Med Virol* 2013;85:620-626.

- 50) **Montpellier C, Wychowski C**, Sayed IM, Meunier JC, Saliou JM, Ankavay M, et al. Hepatitis E virus lifecycle and identification of 3 forms of the ORF2 capsid protein. *Gastroenterology* 2017;pii: S0016-5085(17)36179-6.
- 51) Nair VP, Anang S, Subramani C, Madhvi A, Bakshi K, Srivastava A, et al. Endoplasmic reticulum stress induced synthesis of a novel viral factor mediates efficient replication of genotype-1 hepatitis E virus. *PLoS Pathog* 2016;12:e1005521.
- 52) Tsarev SA, Tsareva TS, Emerson SU, Rippey MK, Zack P, Shapiro M, et al. Experimental hepatitis E in pregnant rhesus monkeys: failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV. *J Infect Dis* 1995;172:31-37.

- 53) Xia J, Liu L, Wang L, Zhang Y, Zeng H, Liu P, et al. Experimental infection of pregnant rabbits with hepatitis E virus demonstrating high mortality and vertical transmission. *J Viral Hepat* 2015;22:850-857.

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